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A Double Staining Method Using SYTOX Green and Calcofluor White for Studying Fungal Parasites of Phytoplankton

Mélanie Gerphagnon, Delphine Latour, Jonathan Colombet, Télesphore Sime-Ngando

LMGE, Laboratoire Microorganismes: Génome et Environnement, UMR CNRS 6023, Clermont Université, Université Blaise Pascal, Aubière, France

We propose a double staining method based on the combination of two fluorochromes, calcofluor white (CFW; specific chitinous fluorochrome) and SYTOX green (nucleic acid stain), coupled to epifluorescence microscopy for counting, identifying, and investigating the fecundity of parasitic fungi of phytoplankton and the putative relationships established between hosts and their chytrid parasites. The method was applied to freshwater samples collected over two successive years during the terminal period of autumnal cyanobacterial blooms in a eutrophic lake. The study focused on the uncultured host-parasite couple *Anabaena macrospora* (cyanobacterium) and *Rhizosiphon akinetum* (Chytridiomycota). Our results showed that up to 36.6% of cyanobacterial akinetes could be parasitized by fungi. Simultaneously, we directly investigated the zoosporic content inside the sporangia and found that both the host size and intensity of infection conditioned the final size and hence fecundity of the chytrids. We found that relationships linking host size, final parasite size, and chytrid fecundity were conserved from year to year and seemed to be host-chytrid couple specific. We concluded that our double staining method was a valid procedure for improving our knowledge of uncultured freshwater phytoplankton-chytrid couples and so of the quantitative ecology of chytrids in freshwater ecosystems.

Many phytoplankton species are susceptible to fungal parasitism. In freshwater lakes, the main parasitic zoosporic fungi belong to the Chytridiomycota (i.e., chytrids). Recent advances in molecular biology have revealed unsuspected fungal diversity in the small size fraction (0.6 to 5 μm) (1), which comprises the dissemination form (i.e., zoospores). Microscopic studies of the reproductive stages of chytrids (i.e., the sporangium) have also revealed the wide diversity of chytrid species in the pelagic zone occurring in lakes throughout the year (2). Chytrids, like some other parasites, are completely dependent on their host for their nourishment and their development, which results in the death of their host (3). Previous studies focused on the impact of fungal parasitism on phytoplankton dynamics and have highlighted the fact that fungal parasitism has had a real impact on the decline of several eukaryotic phytoplankton species, showing that fungal parasitism is potentially implicated in the phytoplankton succession (4–10).

The prevalence and intensity of infection constitute the two parameters classically used to estimate the ecological impacts of fungal parasitism on phytoplankton (11, 12). However, it has been recognized that zoospores are an important source of carbon in freshwater ecosystems and act as a real link between inedible host algae and higher trophic levels (4, 8). It is therefore surprising to find that the accurate quantification of chytrid fecundity (i.e., number of zoospores per sporangium), a key parameter in chytrid life cycles, is often omitted in field studies. As far as we are aware, no study has directly investigated the classical parameters of fungal infectivity alongside the abundance of zoospores or identified any host parameters that could influence them. Nowadays, a precise investigation of these questions requires chytrid cultures (6). However, in freshwater lakes, the phylum of Chytridiomycota is dominated by uncultured fungi (13, 14). This could explain why the effects of various different parameters (light, temperature, and nutrients) on chytrid development have been investigated for only a single host-chytrid couple, *Asterionella formosa-Rhizophydium planktonicum* (6, 15, 16). Moreover, to the best of our knowledge, the survey of the putative relationships between host cell size and chytrid fecundity during a field study has been mentioned only once and even then not directly demonstrated for the host-parasite couple, *Stephanodiscus alpinus-Zygorhizidium* sp. (17).

To overcome the methodological limitations, we propose a double staining method based on a combination of two fluorochromes (calcofluor white [CFW] and SYTOX green) coupled with epifluorescence microscopy to identify, count, and investigate the interactions that exist within uncultured host-parasite couples. We applied our method to the uncultured couple formed by the cyanobacterium *Anabaena macrospora* and one of its chytrid parasites, *Rhizosiphon akinetum*, collected during a field study. We also investigated the prevalence and intensity of infection, host cell size, fungal size, chytrid fecundity, and the putative relationships connecting these parameters in this uncultured host-parasite couple.

MATERIALS AND METHODS

Study site and sample collection. Samples were collected in Lake Aydat (45°39′48″N, 002°59′04″E), a small eutrophic lake (maximum depth [Z_{max}] = 15 m, surface area = 60 ha) with a large catchment area (3×10^4 ha), which is located in the French Massif Central region. Lake Aydat was sampled on 18 October 2010 and 21 October 2011. These sampling periods in two successive years corresponded to the termination of *Anabaena macrospora* bloom events (shown by vertical profiles obtained with a spectrofluorometric probe [FluoroProbe; bbe, Moldaenke, Germany]) at

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Address correspondence to Mélanie Gerphagnon, melaniegerphagnon@hotmail.fr.
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what is known to be a time of peak chytrid infection (18). During both samplings, the sample point was located in the center of the lake, at the maximum depth. Twenty liters of each water layer was sampled using an 8-liter Van Dorn bottle. In October 2010, samples were collected in the surface euphotic layer (estimated by Secchi disk) at 0.5 m. In order to investigate the vertical distribution of chytrid infection, four different depths located in both the euphotic (0.5-m and 2-m) and aphotic (6-m and 9-m) layers were sampled on 21 October 2011. To eliminate the metazoan zooplankton, immediately after collection, the samples were prefiltered through a 150-μm-pore-size nylon filter, poured into clean transparent recipients, and then transferred to the laboratory for processing. The fraction consisting of particles > 150 μm in diameter was checked to make sure that it did not contain any cyanobacterium.

Back in the laboratory, samples were treated (i) to study the host community (triplicate 180-ml aliquots of the raw samples were fixed with Lugol's iodine) and (ii) to investigate the prevalence and intensity of infection and chytrid fecundity using the size-fractionated-community method developed by Rasconi et al. (12). Briefly, 18 liters of the sampled water was concentrated on a 25-μm-pore-size nylon filter. Large (≥25-μm-diameter) phytoplankton cells, including cells of the filamentous cyanobacterium *A. macrospora*, were collected by washing the filter with 0.2-μm-pore-size-filtered lake water, fixed with formaldehyde (2% final concentration), and used to carry out our double staining method described below.

Samples collected in 2011 were used (i) to optimize our method and (ii) to investigate at the same time classical infectivity parameters (prevalence and intensity of infection) and putative relationships between host cell size, final parasite size, and fungal zoosporic content inside a single host-parasite couple described phenotypically as *Anabaena macrospora-Rhizosiphon akinetum*. We used samples collected and fixed in October 2010 to check our method on samples that had been conserved for a year in formaldehyde and to find out whether the relationships found in 2011 were maintained from year to year.

Physicochemical parameters. Water transparency (Z_s) was measured *in situ* using a Secchi disk, and the depth of the euphotic zone (Z_{eu}) was calculated according to the method of Reynolds (48) as follows: $Z_{eu} = 1.7 \times Z_s$. Temperature and dissolved oxygen profiles were obtained using a ProOdO multiparametric probe (Ysi, Germany). A vertical pigment profile was obtained by using a bbe Fluoroprobe (Moldaenke, Germany) (see Fig. S1 in the supplemental material).

Host community analysis. Just one chytrid species, identified phenotypically as *R. akinetum*, was reported in two successive years on akinetes. As a consequence, the host community analysis was focused on the host of this chytrid: akinetes of *A. macrospora*. Triplicate 180-ml aliquots of raw samples were fixed with Lugol's iodine. For each replicate, 5 to 20 ml (depending on the phytoplankton density) was allowed to settle overnight in a counting chamber. The cells were then counted under an epifluorescence microscope (Zeiss Axiovert 200 M) following the classical Utermöhl method. The entire counting chamber was inspected, and *A. macrospora* akinetes were quantitatively analyzed by counting the numbers of immature akinetes (I.A.) and mature akinetes (M.A.). Distinguishing between these two classes is performed on the basis of morphology (the presence of an outer envelope layer is characteristic of mature akinetes), shape (mature akinetes are ovoid whereas immature akinetes are spherical) (19), and size (16 to 23 μm width and 21 to 28 μm length for the ovoid mature akinetes versus 13 to 17 μm diameter for spherical immature akinetes).

Double staining of fungal parasites. Because it was impossible to investigate the number of zoospores inside sporangia of chytrids without staining, as the zoospores are simply invisible (see Fig. 2A and E), we tested a double staining method combining two fluorescent stains to quantify the zoosporic content of sporangia together with the classical infectivity parameters on the same sample. First, we tested the combination of the classical nucleic acid stain DAPI (4′,6′-diamidino-2-phenylindole) (1 $\mu g \cdot ml^{-1}$) with the specific chitinous fluorochrome calcofluor white (CFW [C40H44N12O10S2; Sigma catalog no. F3543]) (final concentration, 2.5% [vol/vol]) as in the original protocol (12) Second, we combined the nucleic acid stain, SYTOX green (Molecular Probes, Invitrogen) and the CFW. Ranges of concentrations (0.1 μM, 0.2 μM, and 0.5 μM) and incubation times (30 min and 1 h) of SYTOX green were tested. CFW was used at a 2.5% (vol/vol) final concentration. CFW was added to the samples either at the same time as SYTOX green (i.e., 30 min or 1 h of incubation) or just 5 min before the observations. After staining, the samples were incubated in the dark at room temperature. The sample-processing procedure is summarized in Fig. 1. The optimal procedure consisted of an incubation time of 55 min with a 0.1 μM final concentration of SYTOX green before addition of CFW followed by a further incubation lasting 5 min.

Microscopic assays. To prevent bleaching, 5 μl of an antifading solution (4:1 mixture of Citifluor and VectaShield; Vector Laboratories) was added before mounting the samples between glass slides and coverslips. The chitin walls stained with CFW were examined using UV excitation (405 nm) (Fig. 2B); zoospore nuclear genomic DNA stained with SYTOX green was explored under blue light illumination (488 nm) (Fig. 2C). Initially, some assays were done with a Zeiss LSM 510 Meta confocal microscope to visualize the zoosporic content inside the sporangia. Confocal microscopy has the advantage of automatically providing numerous images in the *z* axis (one every 0.65 μm), which provided a precise investigation of the entire content of the sporangium and so very good picture quality (in two dimensions [2D] or [3D]). However, the lasers used by confocal microscopy accelerated the bleaching of sample despite the addition of antifading agent, and this limited our ability to measure classical chytrid parameters (prevalence and intensity of infection). Moreover, this equipment is uncommon and expensive. We then applied our staining procedure to the samples and carried out the observations under a Zeiss Axiovert 200 M inverted epifluorescence microscope (Fig. 2). Images of the zoosporic content were acquired at intervals of 0.8 to 1.1 μm and then analyzed using Axiovision 4.1 software. Samples observed using the epifluorescence microscope (Zeiss Axiovert 200 M) displayed a shorter bleaching time, which allowed us to investigate several classical fungal parameters in parallel and thus to survey the relationships between host cell size and parasite fecundity. Because no difference was observed between the relationships established between sporangium size and zoosporic content obtained using these two types of microscopy, the epifluorescence microscopy procedure was subsequently applied to all samples collected.

Chytrid parasitism. Samples were observed between glass slides and coverslips at ×400 magnification under an epifluorescence microscope (Zeiss Axiovert 200 M). To confirm the target of fungal parasitism, 300 akinetes were inspected corresponding to each depth sampled without distinguishing between mature and immature akinetes. From this count, mature akinetes were found to be the only host cells harboring *R. akinetum*, and so 300 mature akinetes were inspected for each depth. Each sample was analyzed in triplicate. Additionally, the biovolume of 100 randomly selected mature infected akinetes and 100 randomly selected mature uninfected akinetes was calculated, assimilating mature akinetes to ovoids (20).

Infection parameters were calculated according to the formula proposed by Bush et al. (11). These parameters include the prevalence of infection of akinetes (Pr_{AK}), i.e., the proportion of individuals in a given population with at least one fixed sporangium, expressed as Pr_{AK} (%) = [(*N*i/*N*) × 100], where *N*i is the number of infected host akinetes and *N* is the total number of host akinetes. The second parameter is the mean intensity of infection of akinetes (I_{AK}), calculated as I_{AK} = Np/Ni, where Np is the number of parasites and Ni is the number of the infected individuals within a host population. The life stage of each chytrid encountered on a cyanobacterium was noted according to Canter (21). Stage 1 corresponded to a zoospore which had just penetrated the mucilage of a living host cell using a fine thread. After this encystment phase, the zoospore discharges its content into the host cell, resulting in a globose structure known as the prosporangium (stage 2). The prosporangium expands

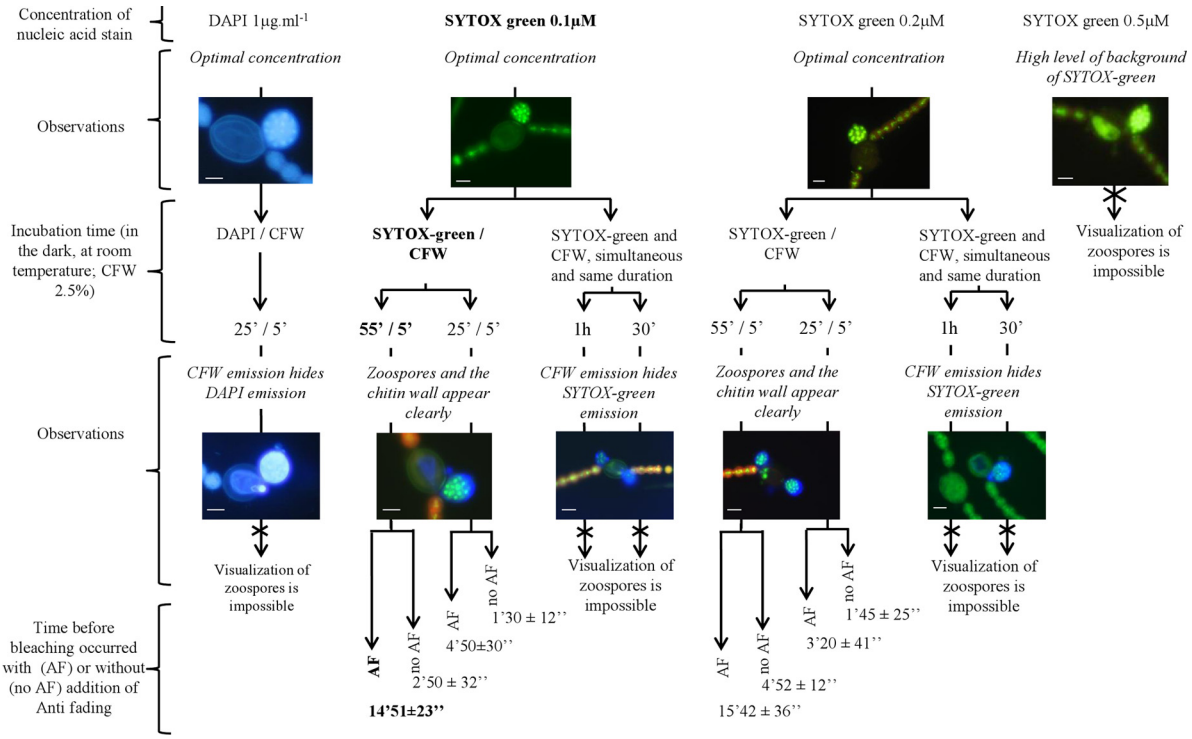

FIG 1 Different concentration and incubation time procedures tested for double staining method and epifluorescence microscopy observation of zoosporic content and sporangia of phytoplankton parasitic chytrid. Optimal procedures are indicated with bold characters. Bars, 10 µm.

into an akinete (stage 3) followed by the emergence of an epiphytic bud corresponding to the primary prosporangium stage (stage 4), which in turn evolves into a mature spherical sporangium surmounted by a papilla (stage 5). The sixth and final stage of the life cycle of *R. akinetum* corresponds to the empty sporangium after deliquescence of the papilla and extrusion of the zoospores.

For each mature and empty sporangium, the biovolume of the host akinete and the biovolume of the sporangia were calculated by assimilating sporangia to spheres and akinetes to ovoids (20). Empty sporangia displaying a deformation of the cell wall were not taken into consideration in investigating relationships between host cell size and final chytrid size. For each mature stage encountered, the fecundity of the chytrid was investigated by analyzing the zoosporic content in the various different images acquired as described above. Because the abundance of mature and empty sporangia encountered during the survey of classical parameters was too low in the samples collected in 2010, 30 mature sporangia were investigated independently from these counts.

Statistical analyses. Because of unequal sample sizes, the nonparametric Kruskal-Wallis test was used, followed by a Mann-Whitney pairwise comparison with the Bonferroni correction to test differences in the biovolumes of akinetes and sporangia at different depths. Spearman's correlation coefficient was calculated to investigate relationships between the biovolume of sporangia or akinetes and the zoosporic content. The abundances of akinetes (mature or immature) were tested using a one-way analysis of variance (ANOVA). All statistical analyses were conducted using PAST, and graphics procedures were performed using Sigma plot 11.0.

RESULTS

Physicochemical parameters. The temperatures and oxygen concentrations presented similar values and profiles in the two successive years. Thermoclines were located at 8 m in both years, with the maximum temperatures obtained in the euphotic layer ([means ± standard deviations {SD}] being 11.7 ± 0.3°C and 12.4 ± 0.2°C for 2010 and 2011, respectively). The euphotic layer was well oxygenated in both years (8.2 ± 0.1 8.8 ± 0.14 mg · liter^{-1} for 2010 and 2011, respectively) (see Fig. S1 in the supplemental material).

The host community. Vertical profiles obtained using the bbe Fluoroprobe revealed the strong predominance of cyanobacteria in Lake Aydat at both sampling dates (from a 72% total concentration of chlorophyll a at 10 m in October 2010 to more than 99.5% at 1 m in October 2011) (see Fig. S1 in the supplemental material). For each year, the cyanobacterial community was heavily dominated by the filamentous blue-green alga *Anabaena macrospora*. A total of 81.6% ± 7.2% of *A. macrospora* filaments presented as either M.A. or I.A.

In October 2010, the density of mature akinetes (M.A.) (3.69 ± 0.7 × 10^5 akinetes · liter^{-1}) was 3 times greater than that of immature akinetes (I.A.) (1.1 ± 0.2 × 10^5 akinetes · liter^{-1}) (Table 1).

In 2011, at all depths, on average the M.A. density was double that of the I.A. (3.4 ± 0.53 × 10^5 M.A. per liter versus 1.65 ± 0.15 × 10^5 I.A. per liter at 2 m, for example). M.A. and I.A. exhibited significantly ($P < 0.001$) higher values in the first three depths than in the deepest depth. No significant difference was noted between the different depths (for the biovolume of mature akinetes; data not shown). Because no infection of I.A. was reported during the first investigation, we subsequently focused solely on mature akinetes.

Staining of chytrids. The zoosporic content of sporangia was stained by DAPI (Fig. 1), but the experiment using the combination with the CFW was unsuccessful, as the two dyes presented

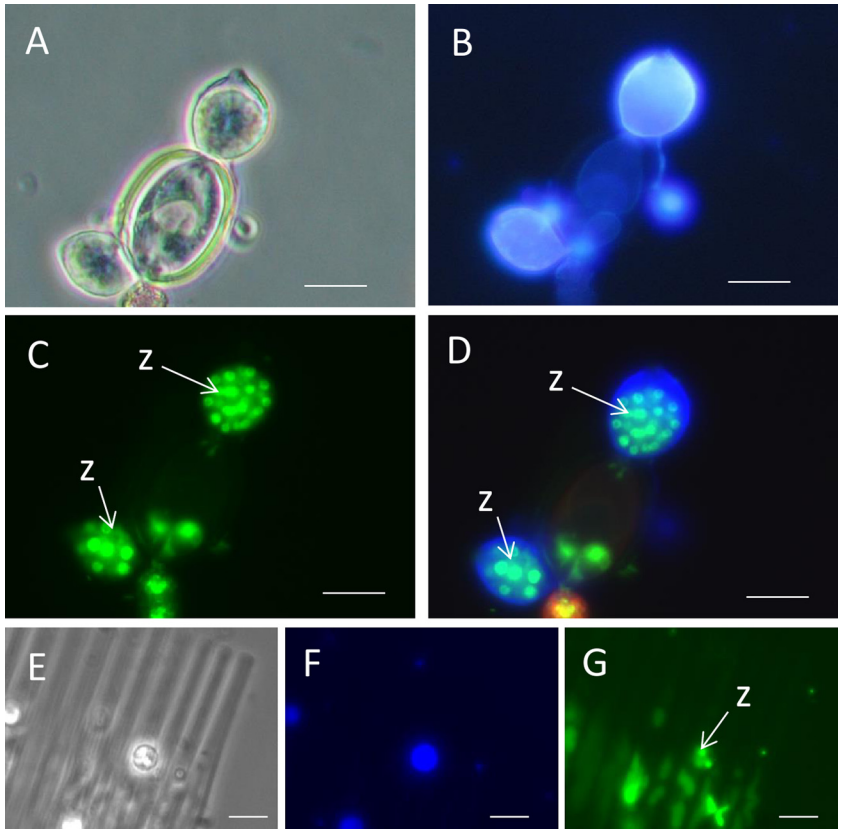

FIG 2 Sporangium of *Rhizosiphon akinetum* (A, B, C, and D) and *Rhizophydium fragilariae* (E, F, and G) stained by the double staining method (CFW and SYTOX green) and excited by white light (A and E), UV light (B and F), blue light (C and G), or both UV light and blue light (D) as observed by optical microscopy. Bars, 10 μm. z, zoospores.

two rather close emission wavelengths. The best staining intensity obtained for the zoospore DNA content was recorded with concentrations of SYTOX green of 0.1 and 0.2 μM (no difference between the results determined with these two concentrations was noted). In contrast, a high level of background "noise" was found at the highest concentration (0.5 μM). Despite the addition of antifading agent, considerable bleaching was observed for incubation times of less than 1 h with SYTOX green (Fig. 1). We therefore

TABLE 1 Biological variables measured in the eutrophic Lake Aydat 18 October 2010 and 21 October 2011[a]

Parameter	Value							
	2010 (0.5 m)		2011 (0.5, 2, 6, and 9 m)					
			Min		Max			
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Density of M.A. ($\times 10^5$ akinete · liter^{-1})	3.69	0.72	2.11[e]	0.28	3.4[c]	0.53	3.3	0.6
Density of I.A. ($\times 10^5$ akinete · liter^{-1})	1.1	0.2	1.19[e]	0.5	1.66[d]	0.15	1.45	0.25
Prevalence of infection by *R. akinetum*	4.6	1	28.2[b]	0.02	36.6[c]	0.02	31.9	3
Intensity of infection by *R. akinetum*	1.2	0.1	1.42[c]	0.03	1.46[d]	0.1	1.44	0.02
No. of *R. akinetum* parasites at life stage 1	3.7	0.5	21.3[b]	6	34[c]	7.8	29.58	7.08
No. of *R. akinetum* parasites at life stage 2	2.3	1.1	40.3[e]	1.15	45[b]	1.5	42.41	11.8
No. of *R. akinetum* parasites at life stage 3	1	1	37[b]	10	45.6[c]	9.2	40.5	8.25
No. of *R. akinetum* parasites at life stage 4	0.3	0.6	1.6[b]	0.5	2[e]	1	2.25	1.35
No. of *R. akinetum* parasites at life stage 5	2.6	0.5	4[d]	3	9.6[c]	3.2	6	3.2
No. of *R. akinetum* parasites at life stage 6	4.33	1.5	14[b]	4.5	19.6[c]	4.5	16.25	4.5

[a] Min, minimum; Max, maximum; SD, standard deviation. See Materials and Methods for an explanation of what the values for prevalence of infection and intensity of infection represent.
[b] Values reported at 0.5 m.
[c] Values reported at 2 m.
[d] Values reported at 6 m.
[e] Values reported at 9 m.

adopted an incubation time of 1 h and a final SYTOX green concentration of 0.1 μM. In contrast to the nucleic acid stain, an incubation time of 5 min was long enough for CFW to stain its target, the chitin wall. However, when it was added along with SYTOX green, the fluorescence emitted by CFW hid the clear fluorescence of zoosporic nuclear genomic DNA (Fig. 1). CFW was therefore added 5 min before the end of staining with SYTOX green. To summarize the procedure finally adopted, samples were incubated in the dark for 55 min with 0.1 μM SYTOX green and then, 5 min before the direct microscopic observations, the CFW was added at a concentration of 2.5% of the stock solution. We considered this procedure to provide optimum conditions for the double staining method proposed in this report.

Prevalence and intensity of infection of mature akinetes. In October 2010, 4.6% ± 1% of akinetes were infected by *R. akinetum* and presented a mean intensity of infection of 1.2 ± 0.1. In the following year, infection reached 36.6% of akinetes, and no significant difference between the results determined at the various depths was found, with the exception of Pr_{AK} at 2 m (36% ± 2%), with a value significantly higher ($P < 0.05$) than the Pr_{AK} value at 0.5 m (28% ± 2%). The mean intensity of infection (1.44 ± 0.02) also appeared to be quite stable throughout the water column. The 6 different life stages of *R. akinetum* reported by Canter (21) were observed during both years and in each water layer. Comparing their abundances did not reveal any significant differences in the values determined for the various depths (Table 1). Each stage of life presented a significantly lower abundance in 2010 than in 2011 ($P < 0.001$).

Relationships between host size, fungal size, and chytrid fecundity. Because the prevalence of infection and, consequently, the abundances of stage 5 (mature sporangia) and 6 (empty sporangia) were too low in 2010, the relationships between host biovolume and the final size of the sporangia was investigated for samples collected in 2011. In 2011, stage 5 and stage 6 represented 5% ± 2% and 12% ± 3% of the total sporangia encountered, respectively. Their abundances were quite uniform throughout the water column; around 6 mature sporangia and 17 empty sporangia were counted in each set of 300 akinetes (Table 1). Their individual biovolumes ranged between 233.5 and 2,213.7 μm^3 and 217.3 and 3,013.09 μm^3, respectively, with no significant difference. This allowed us to investigate the relationship between the biovolumes of host akinetes and sporangia and the zoospore contents, independently of vertical distribution.

Relationship between host biovolume and the final size of the sporangia. Individual biovolumes of parasitized and uninfected akinetes were not significantly different, with average values of 3,694.07 ± 887.8 μm^3 and 3,490.08 ± 823.1 μm^3, respectively (data not shown). Moreover, the biovolumes of host akinetes did not differ significantly according to whether they were parasitized by one or more fungi or depending on the fungal infection stage (stage 5 or 6) (Fig. 3A). In contrast, the biovolumes of *R. akinetum* did present a significant difference (Kruskal-Wallis test, $P < 0.001$) according to the intensity of infection: when the intensity of infection increased, the sporangium size decreased significantly (Mann-Whitney pairwise comparisons, $P < 0.05$) (Fig. 3B). The mean individual biovolume of the mature sporangia was reduced on average by 67.1% for mature and 71.62% for empty sporangia when the intensity increased from 1 to 3 (Fig. 3B). Nevertheless, no differences were found regarding the biovolumes of *R. akinetum* for the different life stages of the chytrids (i.e., whether they were mature or empty) for equal infection intensities (Fig. 3B). As a consequence, we pooled data from these two stages for comparisons to the biovolumes of their host akinetes. The results revealed a significant linear relationship ($y = 94.25 + 0.3923x$; $r^2 = 0.52$; $P < 0.001$) between the akinete size and sporangium size for an intensity of infection equal to 1 (Fig. 4).

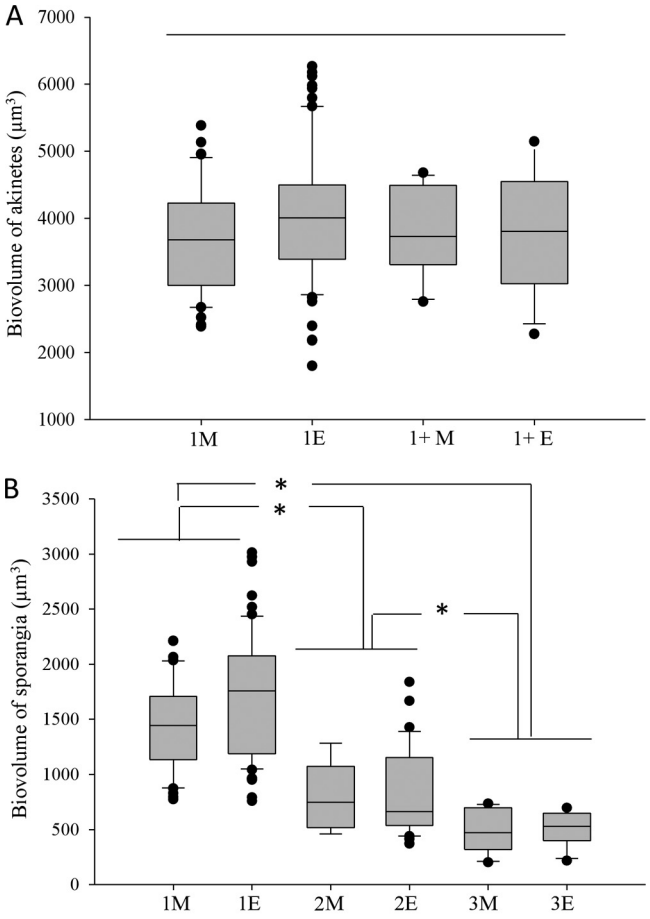

FIG 3 Biovolume of host akinetes (A) and sporangia (B). (A) Akinetes were parasitized by one (1M) or several (1+M) mature sporangia or by one (1E) or several (1+E) empty sporangia. (B) Sporangia of *R. akinetum* were mature (M) or empty (E) and were alone (1M and 1E) or there were two (2M and 2E) or three (3M and 3E) per akinete. Box plots surmounted by a black line indicate no significant difference (Mann-Whitney pairwise comparisons, $P > 0.05$); asterisks indicate significant differences (Mann-Whitney pairwise comparisons, $P < 0.05$). Horizontal lines represent variable medians, boxes delineate the first and the third quartiles, and circles represent potential outliners.

Relationship between the sporangium biovolume and zoosporic content. Our double staining method (SYTOX green combined with CFW) allowed us to investigate the number of zoospores in each mature sporangium. The number of zoospores per sporangium ranged from 6 to 44 (Fig. 5A) and was significantly correlated ($r^2 = 0.65$; $P < 0.001$) to the size of the sporangium (Fig. 5B). Application of our double staining method to samples collected in 2010 and preserved for 1 year in formaldehyde was successful. The number of zoospores per sporangium ranged from 2 to 45, and the numbers found were significantly correlated to the volume of mature sporangia ($r^2 = 0.625$; $P < 0.001$), which did not differ from the 2011 values. From results obtained in 2010 and 2011, we established a conversion factor (CF), calculated as CF =

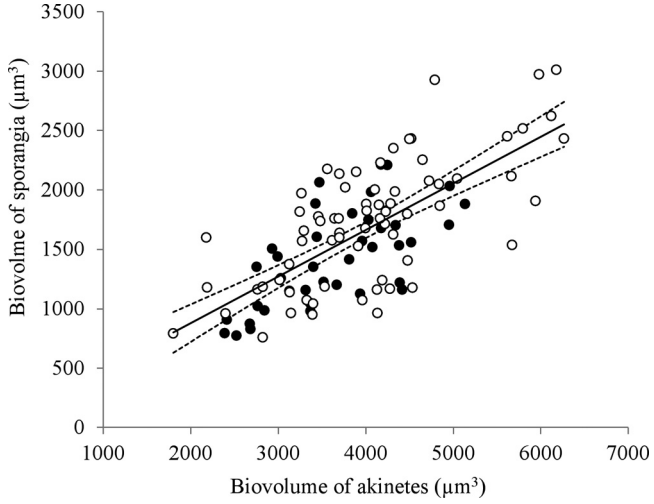

FIG 4 Biovolume of fungal parasite sporangia plotted against the volume of host akinetes. Hollow circles represent empty sporangia. Filled circles represent mature sporangia. Linear regression values and 95% confidence intervals apply to all sporangia ($y = 94.25 + 0.3923x$; $n = 131$; $r = 0.79$; $P < 0.001$).

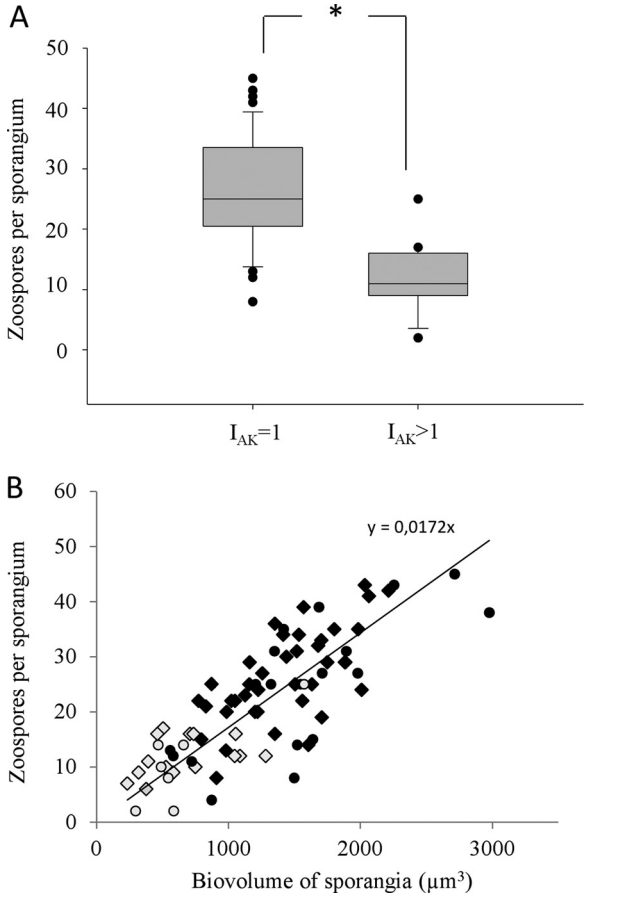

FIG 5 (A) Number of zoospore per sporangium when the intensity of infection of the akinete is equal to 1 ($I_{AK} = 1$) or greater than 1 ($I_{AK} > 1$) (horizontal lines represent variable medians, boxes delineate the first and the third quartiles, and circles represent potential outliners). (B) Comparison of number of zoospores per sporangium plotted against biovolume of sporangia when fungi were present singly (black) or in multiple numbers (gray) per host cell in 2011 (diamonds) and 2010 (circles).

$N_z/V_{M.A.}$, where N_z is the number of zoospores in the mature sporangium and $V_{M.A.}$ is the biovolume of the sporangium (μm^3). We obtained a conversion factor for *R. akinetum* of 0.0172 zoospores per μm^3 of sporangium (Fig. 5B). For the two successive years sampled, no difference was found for the conversion factor or the links established between final fungal size and their fecundity; thus, we assumed that relationships established for the chytrid fecundity were conserved from year to year.

DISCUSSION

The aim of this study was to develop a quick and easy method to explore the relationships existing between chytrid parasites and their phytoplankton host species, including parasite fecundity and the infection parameters that could influence it. To the best of our knowledge, no method has so far been devised that can be used to survey the prevalence and intensity of infection, host cell size, fungal size, and zoospore production simultaneously. To fill this gap, we propose a double staining method based on a combination of two classical fluorochromes: CFW and SYTOX green.

Previous techniques used for the direct investigation of zoospore production required culturing. Bruning (6) assumed that the best way to quantify the zoospore content in mature sporangia accurately was to wait for the sporangium to discharge its contents and then count how many zoospores are released. This method requires having a host-parasite couple that can be maintained under culture conditions. However, in freshwater lakes, Chytridiomycota are dominated by uncultured fungi (13, 14). Chytrids are difficult to culture, and their maintenance under laboratory conditions requires huge efforts (22). Moreover, some traits of parasites, such as zoospore production, could be altered by the culture method, which could modify the natural relationships existing between the host and the parasite (23). Molecular biology methods such as catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) could provide quantitative data without dependence on culture conditions (24). However, such a method is based on the specificity of a probe with rRNA target sequences. This requires DNA sequences of chytrids of interest, which are not always known (and were not known in this study). Moreover, this method is often specific to species, genus, or order (25), while the double staining method proposed in this study could be used on various species of chytrids without any discrimination. Additionally, by binding the ribosomal structures, the CARD-FISH emits a diffuse signal which could exclude a precise quantification of zoospores inside sporangia. Thus, we consider the double staining method proposed in this study to be a good candidate for avoiding the need for cultures and for accurate quantification of the number of zoospores per sporangium, as well as for providing possible species identification of both the chytrid and the host. Calcofluor white (CFW) binds to ß1-4 polysaccharides, such as those found in chitin, which composes the fungal cell wall (26). Rasconi et al. (12) described CFW as the best fluorochrome for studying the morphological diversity of chytrids. Because it is impossible to survey zoospore numbers inside sporangia without using a stain (Fig. 2A), and because the traditional nucleic acid stain, DAPI (4′,6′-diamidino-2-phenylindole), has an emission wavelength (470 nm) rather close to that at which CFW fluoresces (435 nm) (Fig. 1) (27), we combined the CFW with the staining nucleic acid SYTOX green (Fig. 1 and 2). This

stain binds only to the nucleic acid of permeabilized cells, which is why it is commonly used to discriminate between viable and nonviable cells or spores (28, 29). Our samples were fixed (for times ranging from a few hours to 1 year) with formaldehyde, which is known to permeabilize cell membranes (30). SYTOX green was not used here to identify viable cells but was chosen for its capacity to emit green fluorescence (523 nm) when it binds to nucleic acid. Under optimal conditions (incubation time of 55 min with a 0.1 μM final concentration of SYTOX green followed by addition of 2.5% of CFW for a further incubation time of 5 min), the double staining procedure made it possible to visualize the chitin wall at the same time as the zoospore (Fig. 1 and 2D) and did not interfere with identification of host cells and chytrid parasites.

Based on morphological criteria, we identified a host-parasite couple, *Anabaena macrospora-Rhizosiphon akinetum*, sampled on 18 October 2010 and 21 October 2011. *R. akinetum* is a well-known chytrid parasite of cyanobacteria and, more particularly, of the genus *Anabaena* (31–34). It specifically parasitizes the resting spores (i.e., akinetes) of these cyanobacteria. Not only was *R. akinetum* limited to one type of host cell, but it also seemed to be restricted to the fully grown akinetes. Whatever the abundance of *R. akinetum*, no parasite was reported on immature akinetes during the two successive years sampled, whereas mature akinetes were more or less severely parasitized (from 4% ± 1% in 2010 to 36.6% ± 2% in 2011) (Table 1). Canter (21) described the same limitation of infection to mature akinetes in samples collected in a pond in South Bohemia, Czech Republic. Immature and mature akinetes presented two main differences: (i) their macromolecular compositions and (ii) their morphologies. During the akinete differentiation, Sutherland et al. (19) reported a 2-fold increase in total carbon and a 16-fold increase in glycogen, which constitutes the prime energy reserve of zoospores for dispersal (35, 36). These macromolecular changes could explain why chytrids are attracted by mature akinetes. In fact, some authors have proposed a possible chemotactic attraction between host and parasites (37, 38). Moreover, Holfeld (17) has suggested that host size could be a driving factor of infection. That author also reported that infected cells of the diatom *Stephanodiscus alpinus* had a higher median diameter than uninfected cells. This hypothesis could explain why fungi are restricted to mature akinetes. However, no difference in the biovolumes of parasitized and uninfected mature akinetes (data not shown) was found in our study, and so this hypothesis could not be the only explanation. In addition, no correlation was found between host biovolume and intensity of infection. Those last two observations contradict the suggestion that host size exerted a driving force on *R. akinetum* distribution (Fig. 3A).

However, the host size did seem to drive chytrid parasitism by influencing its fecundity. In fact, we pointed out a significant correlation between host cell size and the final parasite size. This phenomenon, currently observed in parasitology (39–41), has been reported just once in the context of the fungal parasitism of phytoplankton (17). Chytrids, like some other parasites, feed on the contents of their host (3), and so their development and final size are closely dependent on these contents. However, host content is limited, and so as infection intensity increases, less content is available for each individual parasite. This could explain why parasite size is significantly lower when akinetes presented as multiple infections (Fig. 3B). With regard to its development, the fecundity of chytrid species was closely related to sporangium size. During maturation, sporangia undergo multiple mitotic divisions and the cytoplasm is totally transformed into several uninucleated zoospores (42, 43). Using the double staining method proposed in this study, we have been able to identify this close relationship in *Rhizosiphon akinetum* and to establish a conversion factor (CF) of 0.0172 zoospores per μm^3 of sporangium for *Rhizosiphon akinetum.* In our study, the values corresponding to the conversion factor (number of zoospores per μm^3 of sporangium) did not significantly differ for the different years (Fig. 5A), depths, or infection intensities. Our results are supported by experimental results reported by Bruning (15) revealing that the CF is independent of light, temperature, or intensity of infection. This established factor affords access to the mean zoospore content in sporangium by the simple measurement of biovolume of sporangia and to the putative number of zoospores that had been released in the environment by the measurement of empty sporangia, thus avoiding the need to count numerous zoospores. The access to the capacity of zoospore production combined with the prevalence of infection may provide interesting avenues of investigation of both the effectiveness of fungal infection and the host susceptibility/resistance profile with respect to fungal parasitism.

Otherwise, the conversion factor obtained for *R. akinetum* was approximately one-tenth of that reported for *Rhizophydium planktonicum* (0.166 zoospores per μm^3 of sporangium), a parasite of the diatom *Asterionella formosa* (6). This marked difference could be due to an underestimation of the number of zoospores per sporangium. However, we assumed that our microscopy protocol avoided misestimating the number of zoospores. Images of the zoosporic content were acquired at 0.8- to 1.1-μm intervals, which was considerably less than the diameter of the fungal zoospores of *R. akinetum* (2.5 to 3 μm) (21). Moreover, no difference in biovolume was found between mature and empty sporangia (Fig. 3B), which confirmed that we were investigating fully grown chytrids and not sporangia involved in the maturation process, which could have been resulted in an underestimation of the zoosporic content. The higher fecundity of *R. planktonicum* in the study conducted by Bruning (6) could have been due to a difference in the sizes of zoospores. Smaller zoospores could mean that *R. planktonicum* can produce more zoospores per μm^3 of sporangium. However, Sparrow (33) reported zoospore diameters of 3 to 3.7 μm and 2.5 to 3 μm for *R. planktonicum* and *R. akinetum*, respectively, which contradicts this hypothesis. Moreover, during our investigations of *R. akinetum*, we had some opportunities to observe another chytrid species, *Rhizophydium fragilariae*, a fungal parasite of the diatom *Fragilaria crotonensis* (Fig. 2E, F, and G). There were too few of the organisms to permit a reliable investigation, but preliminary data revealed a conversion factor that was much closer to that of *R. planktonicum* (0.076 zoospores per μm^3 of sporangium) and approximately 4.5-fold higher than that observed for *R. akinetum.* Kagami et al. (8) have shown that the lipid composition of zoospores is linked to the composition of its host. Diatoms are generally considered to provide better food quality than cyanobacteria (44). In addition, Lord and Roberts (45) reported that food quality could influence the zoospore production of *Lagenidium giganteum*. This hypothesis requires further investigation for confirmation, but it might signify that phytoplankton host food quality could drive the fecundity of their chytrid parasites.

In conclusion, we proposed a double staining method based on a combination of CFW and SYTOX green for counting, identifying, and investigating the fecundity of phytoplankton fungal par-

asites and the putative relationships established between hosts and their fungal parasites. Our simple, quick, and cheap method avoids the need for culture conditions and allows direct investigations of fixed natural samples. We have established that host size does not seem to be a driving force of chytrid infectivity but does influence chytrid fecundity. However, our conclusions are mainly based on the results of field observations. Clearly, further investigations of several chytrid species infecting different host species remain necessary for accurate generalization of such relationships. Additionally, we assume that our method presents some limitations concerning the quantification of free zoospores. Molecular biology, i.e., CARD-FISH, could be a good complementary tool for chytrid species for which ribosomal DNA (rDNA) sequences are known (25). Nevertheless, our method does make it possible to investigate zoospore production by chytrid species. Thus, the ability to access the production of zoospores of each chytrid species could provide interesting data for quantifying the carbon input of each species in a mixed fungal community. This could be very useful for authors trying to model the effects of parasitism in freshwater ecosystems (2, 4, 46, 47).

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